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Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that *flaA* but not *flaB* is required for invasion

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The role of the *Campylobacter jejuni* flagella in adhesion to, and penetration into, eukaryotic cells was investigated. We used homologous recombination to inactivate the two flagellin genes *flaA* and *flaB* of *C.jejuni*, respectively. Mutants in which *flaB* but not *flaA* is inactivated remain motile. In contrast a defective *flaA* gene leads to immotile bacteria. Invasion studies showed that mutants without motile flagella have lost their potential to adhere to, and penetrate into, human intestinal cells *in vitro*. Invasive properties could be partially restored by centrifugation of the mutants onto the tissue culture cells, indicating that motility is a major, but not the only, factor involved in invasion.

Key words: bacterial invasion/*Campylobacter*/electroporation/flagellin/virulence factor

Introduction

Diarrhoeal diseases present an ongoing threat to human health (Snyder and Merson, 1982). The Gram-negative spiral bacterium *Campylobacter jejuni* is a major cause of enteritis in man, both in developing and developed countries. *C.jejuni* is often found in healthy mammals and birds (Skirrow, 1990), which serve as epidemiological reservoirs from which the bacteria are transferred to man, usually via food or contaminated water.

Little is known about the molecular biology of the pathogenesis of campylobacter. We have focused on the flagellum, which has been recognized as one of a number of potential virulence factors. This flagellum is unsheathed and its filament consists solely of flagellin. In *C.jejuni* strain 81116 flagellum synthesis is switched on and off, a phenomenon called phase variation (Caldwell *et al.*, 1985). Both phases, the wild-type and Fla$^-$, are relatively stable and could therefore be used in attachment studies *in vivo* and *in vitro*. These studies suggested that flagella are necessary for intestinal infection of mice and hamsters and for attachment to eukaryotic cells (Morooka *et al.*, 1985; McSweegan and Walker, 1986; Aguero-Rosenfeld *et al.*, 1990).

In *C.jejuni* strain 81116 two copies of the flagellin gene, *flaA* and *flaB*, are present in a head-to-tail configuration. Both genes contain 1731 bp and show 95% sequence identity. Only expression of *flaA* was detected (Nuijten *et al.*, 1989, 1990). *Campylobacter coli* has a similar organization of the flagellin genes. However, *flaA* and *flaB* of *C.coli* are less similar, and both are expressed in the same cell (Logan *et al.*, 1989; Guerry *et al.*, 1990).

We have concentrated on the possible differences in the role of the two flagellin genes of *C.jejuni* during invasion i.e. adherence followed by penetration, into INT-407 cells, a cell line derived from human intestinal tissue. To investigate the function of both flagellin genes separately, we developed a method to inactivate the genes. This inactivation was achieved by the introduction of a kanamycin-resistance gene into one or both flagellin genes. This allowed us to study the effects of both genes on motility and invasion. Our results show that only the product of the *flaA* gene gives rise to functional flagella that are required for invasion of INT-407 cells.

Results

Construction of flaA and flaB mutants

To study the role of the two flagellin genes of *C.jejuni* in motility and penetration, we have developed a simple method to inactivate both genes together and separately. For the inactivation of *flaA* we constructed pTNS # A, a Bluescript construct in which a segment of the *flaA* gene is interrupted by a kanamycin-resistance gene (Figure 1A). A similar construct with a disrupted *flaB* gene, pTNS # B, is shown in Figure 1B. The constructs were introduced directly into *C.jejuni* 81116 by electroporation. The cloning vectors used were unable to replicate in *C.jejuni* and selection for kanamycin resistance yielded recombinants in which *flaA*, *flaB* or both genes were disrupted.

DNA isolated from 20 kanamycin resistant *C.jejuni* mutants was digested with the restriction endonuclease *Eco*RV, blotted and hybridized with respectively a probe specific for the Bluescript vector, for the kanamycin-resistance gene and for the flagellin sequences. None of the recombinants contained Bluescript sequences. Four restriction fragment patterns were found with the other two probes (Figure 2). Since *flaA* and *flaB* show 95% sequence identity, several recombination events are possible. Four possibilities, leading to recombinants R1 to R4 are outlined in Figure 1. In R1 two recombinations have occurred within *flaA*, leaving *flaB* undisturbed. In R2 recombinations have occurred in both *flaA* and *flaB*, leading to a large deletion. Homologous recombination of pTNS # B with *flaB* sequences resulted in a mutated *flaB* and an intact *flaA* gene (R3); whereas recombinations within *flaA* resulted in a mutated *flaA* with an intact *flaB* (R4).

The restriction patterns obtained after hybridization were consistent with the four given recombinations. Recombinants R1 and R2 were found after electroporation of pTNS # A and R3 and R4 after electroporation with pTNS # B.

Flagellin gene expression

Bacterial proteins present in whole cell extracts derived from the wild-type, the phase variant Fla$^-$ and the recombinants R1 to R4 were analysed by Western blotting. A polyclonal antiserum and a monoclonal antibody CF17, that are specific for both flagellin A and B protein, detect flagellin in all lanes except those containing the Fla$^-$ phase variant and R2 (Figure 3A and B). With a monoclonal antibody CF1, which is specific for the *flaA* gene product, only the wild-type strain and mutant R3 gave a signal (Figure 3C). A polyclonal antiserum against the major outer membrane protein was used to demonstrate that equal amounts of protein were present in all lanes (Figure 3D). These results confirm that R2 has no functional flagellin gene and that *flaA* is inactivated in R1 and R4. Apparently, *flaB* is expressed in mutants R1 and R4.

Motility and flagellum formation

The production of flagellin does not necessarily result in flagellum formation or motility. In order to test whether the

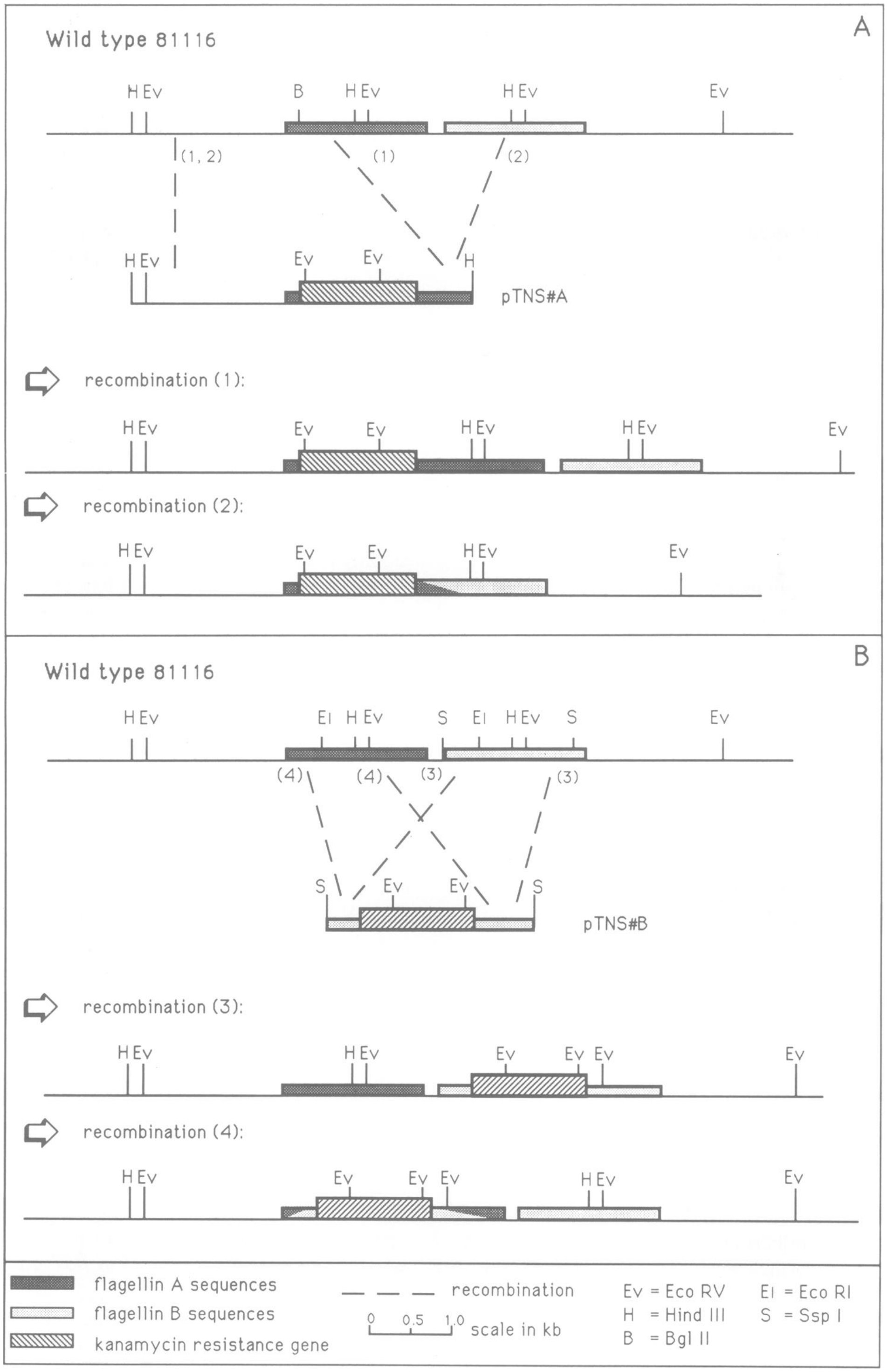

Fig. 1. Schematic representation of recombinational events after introduction of pTNS # A (**A**) or pTNS # B (**B**) into *C.jejuni* 81116. Relevant restriction endonuclease sites are indicated.

flagellin produced by the different recombinants was assembled into flagella filaments, we determined the presence and length of flagella by electron microscopy (Figure 4). The motility of the mutants was determined by dark field microscopy and by colony size in semi-solid thioglycollate medium. Wild-type and R3, which express the *flaA* gene carry long flagella and are motile. Mutant R1 which is defective in *flaA*, but has an intact *flaB* gene, is non-motile and carries short flagella which apparently are unable to confer motility. Table I summarizes the data. There is a clear correlation between motility and the expression of the *flaA* gene.

Invasion of INT-407 cells

C.jejuni strain 81116 is able to associate with and to penetrate into tissue culture cells like many other strains of *C.jejuni* and *C.coli* (Manninin *et al.*, 1982; Newell and Pearson, 1984; De Melo *et al.*, 1989; Konkel and Joens, 1990). Penetration of *C.jejuni* 81116 into INT-407 cells is more efficient than into HEp-2 or HeLa cells. The wild-type and the non-motile aflagellate variant Fla$^-$ differed remarkably in their ability to penetrate into INT-407 cells. The penetration of the Fla$^-$ variant measured after 4 h was 5000 times lower than that of the wild-type strain (Figure 5A). Due to the low level of penetration the results of the Fla$^-$ variant were less reproducible.

The availability of recombinants R1, R2 and R3, lacking an active *flaA*, *flaB* or both genes, made it possible to assay the role of these genes in adhesion followed by penetration into INT-407 cells. The time course of penetration is shown in Figure 5A. Only R3 showed the same penetration as the wild-type strain. The penetration of the other mutants was at least 100 times lower than that of the wild-type strain but slightly better than the Fla$^-$ variant.

To investigate whether motility rather than the *flaA* gene product is required for invasion, we tried to mimic motility by centrifugation of the bacteria onto the INT-407 cells. This treatment raises the level of penetration by R1, R2 and Fla$^-$ considerably immediately after centrifugation; the level of maximum penetration increases 30-fold or more. Penetration by the wild-type and R3 remained the same (Figure 5B). These results suggest that motility, which can be mimicked by centrifugation, is an important factor for invasion.

The possibility that the flagella possess adhesive properties was investigated by competition experiments. Flagella preparations of wild-type, R1 and R3 were released by shearing, purified by sucrose gradient centrifugation and checked by electron microscopy. These preparations were added to INT-407 cells 2 h prior to infection with 81116 wild-type, R1 or R3 bacterial suspensions. No effect on penetration, positive or negative, was observed compared to controls (results not shown). From this we conclude that flagella probably do not have specific adhesive properties that can be blocked by the addition of excess flagella.

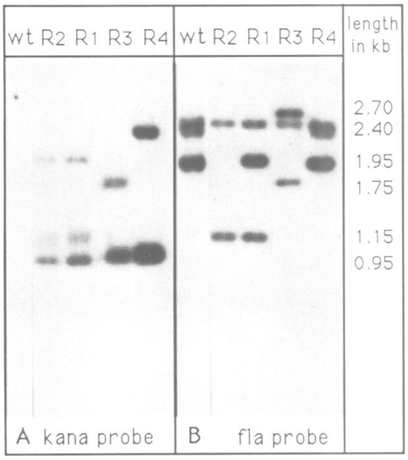

Fig. 2. Characterization of recombinants obtained by electroporation of *C.jejuni* 81116 with pTNS # A and pTNS # B. Autoradiograph of a Southern blot hybridized with probes for the kanamycin-resistance gene (**A**) and a flagellin gene (**B**). Genomic DNA was digested with the restriction endonuclease *Eco*RV. The lengths of the fragments are indicated. Wt: DNA isolated from wild-type 81116; R1 to R4 indicate that the length of the fragments corresponds with the expected recombinants outlined in Figure 1. R1 and R2 were the results of electroporation with pTNS # A; R3 and R4 with pTNS # B.

Discussion

Introduction of DNA into campylobacter has been successful previously. The transfer of shuttle vectors from *E.coli* to *C.jejuni* by conjugation has been demonstrated by Labigne-Roussel *et al.* (1987); the use of suicide vectors results in homologous recombination (Labigne-Roussel *et al.*, 1988; Guerry *et al.*, 1990). Miller *et al.* (1988) developed a method to introduce DNA in campylobacter by electroporation, but

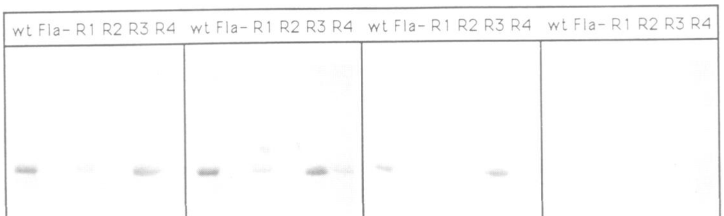

Fig. 3. Western blot of an SDS–polyacrylamide gel stained with polyclonal anti-flagellin serum (**A**), monoclonal anti-flagellin CF1 antibody (**B**), monoclonal anti-flagellin antibody CF17 (**C**) and polyclonal anti-major outer membrane protein serum (**D**). Protein samples are indicated; wt: wild-type 81116; Fla$^-$ is a phase variant lacking flagella. R1 to R4 are as indicated in Figures 1 and 2.

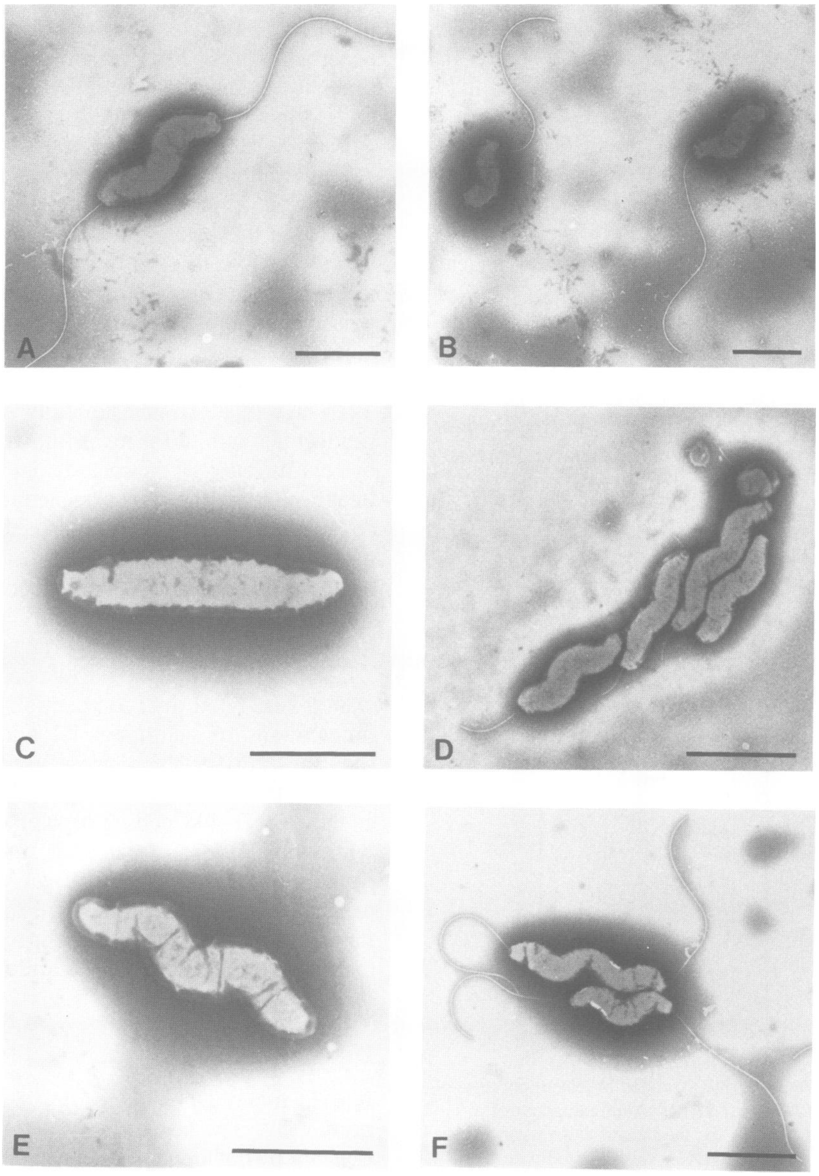

Fig. 4. Electron micrographs of *C.jejuni* 81116 variants and recombinants. The panels show: wild-type (**A** and **B**), Fla$^-$ (**C**), R1 (**D**), R2 (**E**) and R3 (**F**). The bar represents 1 μm.

Table I. Characterization of *C.jejuni* 81116 derived variant and recombinant strains

Strain	Flagellin genotype	Flagellin phenotype	Motility	Flagella in EM
81116 wild-type	A+ B+	A+ B−	+	long
81116 Fla$^-$	A+ B+	A− B−	−	none
81116 (R1)	A− B+	A− B+	−	short
81116 (R2)	A− B−	A− B−	−	none
81116 (R3)	A+ B−	A+ B−	+	long
81116 (R4)	A− B+	A− B+	−	N.D.

attempts to electroporate DNA isolated from *E.coli* into *C.jejuni* did not succeed. The discrepancy between their results and ours may be explained by strain-dependent modification of *E.coli* DNA by campylobacter. The method of electroporation described in this paper is simpler than conjugation and does not require extra cloning steps in suicide vectors.

The finding of a flagellin B product in recombinants R1 and R4 was surprising, since we were unable to detect a transcript of *flaB* in the wild-type strain 81116 (Nuijten *et al.*, 1990). Either there is a low level of expression of *flaB* in wild-type, or else the level of *flaB* expression is increased in the recombinants. This latter possibility would imply a negative regulation of flagellin A on the transcription of *flaB*

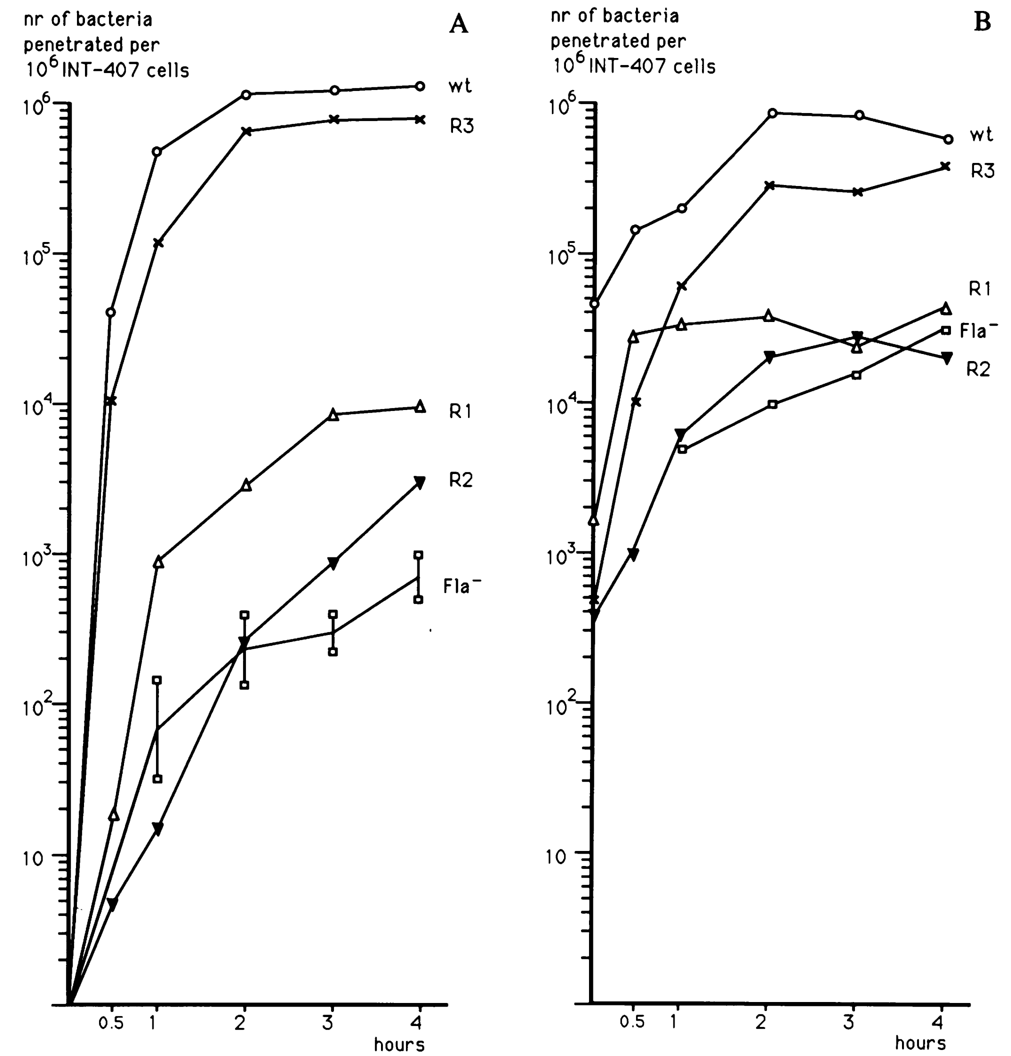

Fig. 5. Time course of penetration to INT-407 cells by wild-type *C.jejuni* 81116; recombinants R1, R2, R3 and phase variant Fla⁻. Bacteria were added to the cells at 0 h (panel **A**). In panel **B** bacteria were centrifuged on the cells from −15 to 0 min. The values for Fla⁻ (A) are mean values of independent experiments.

in wild-type. A possible promoter activity on the kanamycin-resistance gene insert that acts on *flaB* can be excluded, since the insert is present in inverse orientations in R1 and R4 (Figure 1). Extensive RNA analyses of the wild-type and recombinants are in progress, but the detection of flagellin messenger RNA is not without difficulties.

Apparently the flagellin B protein can be assembled into filaments, although these flagella are short and do not confer motility. A low level of expression of *flaB* may be insufficient for full length assembly, or the minor sequence differences between flagellin A and B are responsible for the inappropriate assembly or the instability of the filament.

The importance of flagella as potential virulence factors has been demonstrated for other bacteria. Fla⁻ variants of *Pseudomonas aeruginosa* have a diminished virulence in animal models (Drake and Montie, 1988). In this case motility itself seems to be important since immotile Fla⁺ variants have lost virulence. For *Vibrio cholerae* flagella, but not necessarily motility, are important for virulence and invasion (Guentzel and Berry, 1975; Freter and Jones, 1976; Attridge and Rowley, 1983).

The role of the flagellum of *C.jejuni* during attachment and invasion is clearly demonstrated with the different recombinants. The results of the penetration assays show that the presence of a flagellum consisting of the flaA protein is essential for invasion of INT-407 cells, whereas flagellin B protein is not required. Penetration levels of the immotile mutants and the phase variant are much lower than those of the motile bacteria. We have mimicked motility by centrifuging the bacteria onto the cells. This treatment has an immediate effect on penetration levels, suggesting that motility is important for invasion. Low level penetration is, however, possible without flagella. This is in agreement with earlier data indicating that factors other than the flagella are involved in invasion: namely LPS (McSweegan and Walker, 1986; Fauchère *et al.*, 1989; Konkel and Joens, 1989) and proteins in the range of 26 to 32 kd (De Melo *et al.*, 1989; De Melo and Pechère, 1990). Preliminary data from our own laboratory show that the addition of 30 μg/ml chloramphenicol 30 min prior to infection results in a 95% inhibition of penetration of *C.jejuni*. Chloramphenicol is not toxic for eukaryotic cells and does not affect campylobacter motility, suggesting that bacterial protein synthesis is needed for penetration. Some of these proteins may be involved in the attachment to cells rather than in the penetration. McSweegan and Walker (1986) have reported adhesive properties of flagella preparations, but these could not block bacterial adherence. We have confirmed that the addition of excess flagella does not affect the level of penetration, and therefore we conclude that flagella probably do not have specific adhesive properties.

The role of the flagellum in pathogenesis has not yet been proved directly. The recombinants obtained in this study open the possibility to test this role in animal models (mouse, hamster) or in human volunteers. Finally it will now be possible to construct campylobacter strains differing only in a very short segment of the genome e.g. an epitope recognized by a monoclonal antibody. With these strains it will be possible to assess the role played by natural transformation in the evolution of campylobacter in nature. Wang and Taylor (1990) have shown that natural transformation of campylobacter is possible. In *Neisseria gonorrhoeae* such a mechanism appears to play a role in antigenic variation (Seifert *et al.*, 1988).

Materials and methods

Bacteria, plasmids and culture conditions

The origin and culture conditions of *C.jejuni* strain 81116 have been described (Nuijten *et al.*, 1989). A phase variant lacking flagella and motility (Fla$^-$) and DNA of plasmids pIVB3-300 and pIVB3-304 were provided by P.J.M.Nuijten (Nuijten *et al.*, 1989, 1990). Plasmid pILL550 (Labigne-Roussel *et al.*, 1987) was given by A.Labigne-Roussel. Bluescript vector pBluescript II KS(+) was purchased from Stratagene (La Jolla, CA) and grown in *E.coli* PC2495 (Phabagen Collection, Department of Molecular Cell Biology, University of Utrecht).

Construction of flagellin gene fragments containing a kanamycin-resistance gene

For the construction of pTNS # A we used pIVB3-304 (Nuijten *et al.*, 1990), which contains the first 0.7 kb of *flaA* with a unique *Bgl*II site. A kanamycin-resistance gene was inserted into this *Bgl*II site. We used the gene of campylobacter origin present in pILL550 (Labigne-Roussel *et al.*, 1987) on a 1.4 kb *Hin*dIII−*Eco*RI fragment. The resulting pIVB3-304 derivative was named pTNS # A. Similarly pTNS # B was constructed from a 1.6 kb *Ssp*I fragment of pIVB3-300 containing *flaB* sequences (Nuijten *et al.*, 1990), in which the *Eco*RI−*Hin*dIII fragment was replaced by the above mentioned fragment of the kanamycin-resistance gene.

Electroporation

Electroporation was performed as described by Miller *et al.* (1988) with some modifications. *C.jejuni* 81116 was grown on saponine agar plates for 18 h at 42°C and cells were harvested in 1 ml of ice-cold 15% glycerol−272 mM sucrose, washed and resuspended to 10^{12} cells/ml in the same medium. Aliquots were frozen and kept at −80°C until used. 50 μl of cell suspension was mixed with 0.5−5.0 μg DNA in water. Electroporation was performed with a Bio-Rad Gene Pulser with pulse controller in 0.56 mm gap cuvettes (Biotechnologies and Experimental Research Inc., San Diego, CA) at 0.7 kV, 25 μF and 600 Ω. This leads to time constants ranging from 3.5 to 6.5 ms. Cells were recovered in 200 μl heart infusion broth (Difco) and poured onto heart infusion broth agar plates. After 5 h of regeneration at 37°C in an atmosphere of 5% O_2, 10% CO_2 and 85% N_2 gas, cells were harvested and plated onto saponine agar plates supplemented with 30 μg/ml kanamycin. To determine the number of viable bacteria, 10^{-5} and 10^{-6} dilutions were plated on saponine agar plates.

Isolation of DNA and Southern blot analyses

Plasmid DNA was isolated from *E.coli* by the method of Birnboim and Doly (1979). Genomic DNA was isolated from *C.jejuni* as follows: 10^{10} cells were harvested, washed once in GTE (50 mM glucose, 25 mM Tris−HCl pH 8.0, 10 mM EDTA) and incubated in 600 μl GTE with 3 mg/ml lysozyme for 30 min on ice. Cells were lysed in 0.5% w/v SDS, and digested with 3 μg proteinase K at 45°C. Protein was removed by phenol extraction and the DNA was precipitated with ethanol. 5−10 μg of DNA was digested with *Eco*RV as indicated by the supplier (BRL) and fragments were separated by electrophoresis in 1% agarose gels in TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3) Southern blotting to Hybond-N filters (Amersham), labelling of the probes and hybridizations were performed by standard methods (Sambrook *et al.*, 1989). As a probe specific for the flagellin gene we used the two 1.2 kb *Bgl*II−*Pst*I fragments of pIVB3-300 (Nuijten *et al.*, 1990). As a kanamycin-resistance gene probe the 1.4 kb *Eco*RI−*Hin*dIII fragment from pILL550 (Labigne-Roussel *et al.*, 1987) was used and as a Bluescript probe the complete vector was labelled and used.

Western blots

Whole cell extracts were boiled in the presence of 63 mM Tris−HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol. Samples containing 5 μg protein were electrophoresed in 10% polyacrylamide gels according to Rottier *et al.* (1981). Western blots were produced by electroblotting onto nitrocellulose (BA85, Schleicher and Schuell). Filters were incubated with polyclonal antisera raised against denatured flagellin or major outer membrane protein of *C.jejuni* 81116 (Nuijten *et al.*, 1989), both diluted 1:2000 or monoclonal antibody CF1 (Newell, 1986) and CF17 (Nuijten *et al.*, 1991) diluted 1:250. CF17 reacts with both the *flaA* and *flaB* gene products; CF1 is specific for flagellin A. Antibody binding was detected with alkaline phosphatase-linked goat anti-rabbit or goat anti-mouse antibody (Promega) with nitrobluetetrazolium and bromo-chloro-indolylphosphate as a substrate.

Invasion of C.jejuni into eukaryotic cells

Intestinal cells 407 (INT-407), derived from human embryonal tissue of the jejunum and ileum, were obtained from Flow Laboratories. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and containing 100 International U/ml penicillin and 100 μg/ml streptomycin. The penetration assay was performed in 35 mm tissue culture dishes. Confluent monolayers (~10^6 cells) were washed 30 min prior to infection with DMEM without supplements. A bacterial suspension in DMEM was added to each dish to give a multiplicity of infection of 1000 bacteria per cell. Infected monolayers were incubated at 37°C for 1, 2, 3 and 4 h in 5% CO_2:95% air to allow bacteria to adhere to the cells. After these periods monolayers were washed 5 times with DMEM and reincubated for another 3 h with DMEM containing 250 μg/ml gentamycin. This treatment kills extracellular bacteria, while internalized bacteria are protected since the antibiotic does not enter the cells. Following this second incubation, the monolayers were washed three times with PBS and lysed in 0.5% Triton X-100. The suspensions were diluted and plated onto saponine agar medium and the number of colony forming units was calculated. In some experiments bacteria were brought in contact with the cells by centrifugation of the tissue culture dishes at 600 *g* for 15 min.

Flagella preparations were obtained by shearing 2.5 ml of a bacterial suspension of wild-type and recombinants R1 and R3 (10^{11} cells/ml) in a syringe and centrifugation at 100 000 *g* in 10% sucrose. The pellets were resuspended and checked for the presence of flagellin filaments by electron microscopy. 0.2 ml of these flagella preparations (0.2 mg of flagellin, derived from 8×10^{10} cells) were added to INT-407 cells 2 h prior to infection with 10^9 bacterial cells.

Electron microscopy and motility assay

Bacteria were grown on saponine agar plates for 24 h at 42°C and suspended in 2% bovine serum albumin in PBS. The bacteria were incubated on 200 mesh copper grids coated with Pioloform (Bio-Rad Inc., CA) stained in 1% tungstophosphoric acid (Merck) for 2 min and examined in a Philips EM 201 electron microscope.

The motility of the campylobacter mutants was confirmed by dark field microscopy and by colony size in semi-solid thioglycolate medium (Caldwell *et al.*, 1985).

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